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99	To the personnel of Dept. of Virology, add Postgraduate Student: Hisashi Ogura (小 倉 寿)

DEPARTMENT OF EXPERIMENTAL THERAPEUTICS

GENERAL SUMMARY

The overall objective of our research proposal is the development of fundamental information for delivery of more effective anticancer drugs to cancer patients. Research by which we propose to achieve this objective are centered on the following subjects:

1) Antitumor activity and the action mechanism of β-hemolytic streptococcus or its preparation, OK-432 (NSC-B116209)

Our present studies had their starting point in the study of the effect of RNA on β-hemolytic streptococcus. In 1939, a notable finding was presented by Okamoto of this university that yeast RNA in culture significantly enhanced the formation of streptolysin S (SLS), one of the streptococcal hemolysins. Subsequently, much biological and boichemical data on the nature and formation mechanism of SLS have been presented. In the meantime, we intended to carry on experiments concerning the effect of hemolytic streptococci on tumor cells.

Soon afterward, we uncovered the interesting fact that short incubation of the cocci and tumor cells in simple medium resulted in the production of SLS, and the tumor cells exposed to the cocci were not able to produce growth *in vivo*. In collaboration with the Dept. of Pharmacology, School of Medicine Kanazawa University, evidences have been accumulated to show that the hemolytic streptococci producing SLS is characterized by the property of exhibiting antitumor activity on various animal tumors. On the basis of these findings, experiments in this line of work further developed to establish the conditions which make it possible to produce a useful streptococcal preparation with potent antitumor activity. PC-B-45 and its lyophilized product (OK-432) is a new type of anticancer drug produced with this history for a background.

Recently, there has been a surge of interest in Europe, the United States, and Japan in the use of BCG, Corynebacteria and polysaccharides as a nonspecific immunopotentiator in the treatment of cancer or leukemia. However, OK-432 seems to be different from BCG and others, because OK-432 exhibits two different antitumor effects on tumor growth, namely, a direct pharmacological effect and a host-mediated effect with probable involvement of immune response. Although, OK-432 may be very promising for use as an immunopotentiator in rotation of immunochemotherapy, the more precise investigations on its role in enhancing the host immune response should now be planned.

2) Screening for new class of potent antitumor agents, including synthetic compounds and naturally occurring substances

In early studies, 2-(2-hydroxyphenyliminomethyl)-4-*n*-hexyl-

phenol (HP) was found to be effective against some kinds of experimental animal tumors. Afterward, the modification of HP in structure-activity relationships was followed by a more active compound, 2-(2-hydroxy-5-*n*-hexyl)-8-quinolinol-4-carboxylic acid (HQ II), which has both functional groups, ligand and an aliphatic carbon chain in its molecule. Considering the data obtained in cell kinetic analysis, the antitumor activity of HQ II was quite characteristic, indicating that it might be a compound belonging to S-phase specific with self-limiting agent. At present, in order to develop a valuable antitumor agent, preparation of HP and HQ II analogs is in progress, testing inhibition of DNA synthesis, iron-chelate forming capacity, and damage of the cell membrane as criteria of the modification.

Another proposal was made by planning for *s*-triazine derivatives. Triethylenemelamine (TEM), a known alkylating agent, is a synthetic antitumor agent having a *s*-triazine ring and three aziridyl groups in its structure. It was recently reported that hexamethylmelamine with a structure which closely resembles that of TEM was also effective against human cancer in the lung, uterus, and testis. In this sense, a number of thiourea and carbamate derivatives of *s*-triazine are being submitted to our screening programs. Up to the present, none of the compounds entering clinical trials has found a place among the standard chemotherapeutic agents.

On the other hand, it has been known that some marine substances from endogenous origin are toxic for mammalian red cells or capable of suppressing the growth of animal tumors. In the search for active substances from marine sources, particularly invertebrate animals, it was of great interest that the coelomic fluid of the sea urchin was markedly cytotoxic for transplantable tumor cells as well as animal red cells, and that the drug-resistant cell line of Yoshida sarcoma was found to be less susceptible to the coelomic fluid than the original cell. These findings may present a tool for analyzing the receptor site of the tumor cell membrane or make it possible to obtain active factor(s) to suppress the tumor growth *in vivo*.

3) Isolation and properties of colistin-induced hemolysin from β-hemolytic streptococcus

In the experiments to examine the influence of various antibiotics against streptolysin S (SLS) - forming and antitumor properties of β-hemolytic streptococcus, it was eventually observed that when colistin, a peptide antibiotic, was added to the reaction mixture of the cocci and oligoribonucleotide in the defined medium, the supernatant exhibited very high hemolytic potency. Although the mechanism of the induction of potent hemolysin activity is still obscure, the results obtained in the present study suggested that high hemolytic activity might be caused either by a potent unknown hemolysin other than SLS and streptolysin O, or by labile complexes of SLS and colistin.

These works were supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

ABSTRACT

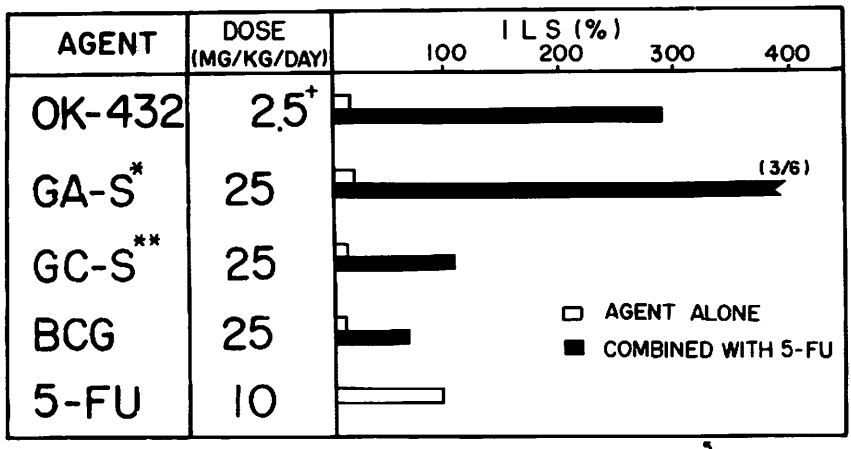
(44) **Antitumor activity and the action mechanism of β-hemolytic streptococcal preparation.**

S. Koshimura, T. Ujiie and K. Ryoyama

OK-432 (NSC-B116209), which is a new antitumor preparation derived from Group A streptococcus, has been shown to be active with a broad antitumor spectrum, and its efficacy in clinical trials has also been evaluated. Although the data on the action mechanism of this preparation is not so sufficient as to explain the characteristic properties, it could be assumed that it was related to the beneficial influence on host immune responses as well as to the direct action on tumor growth. In order to obtain further information on the antitumor action of OK-432, experiments in planned protocols for OK-432 alone or in combination with the other anticancer drugs are being carried out using L1210 mouse leukemia and the other transplantable animal tumors.

The treatment with OK-432 alone was found to be entirely ineffective for L1210 leukemia in any treatment schedules. However, the combined use of OK-432 and sub-optimal dose of some antimetabolites, such as 6-mercaptopurine, 5-fluorouracil, or cytosine arabinoside, was very effective, resulting in a remarkable increase of a life-span of BDF_1 mice bearing the leukemia. The increase of life-span (ILS) produced by the combination therapy was 2-5 times greater than that produced by optimal single-agent therapy, and the therapeutic synergism

ANTILEUKEMIC EFFECT BY COMBINATION OF 5-FU WITH SOME OF BACTERIA



ANIMAL: BDF1 (♀), 6-WEEK-OLD. INOCULUM: L1210 CELLS 2×10^5/HEAD, IP.
TREATMENT: DAYS 1-7, IP.
* GA-S: GROUP A STREPTOCOCCI (SU), HEATED AT 50°C FOR 30 MIN.
** GC-S: GROUP C STREPTOCOCCI, HEATED AT 50°C FOR 30 MIN.
+ WEIGHT OF STREPTOCOCCAL CELLS CONTAINED IN 25KE OF OK-432.

might be dependent upon the administration route and dosage of OK-432 and antimetabolite to be used. Group C streptococcus and BCG were not totally effective in this respect (see Figure). This synergistic effect could not be expected against 5-fluorouracil-resistant L1210 subline, and was abolished to a certain degree either by the pretreatment of the animal with large doses of immunosuppressive agents, such as azathiopurine, or by whole body X-ray irradiation.

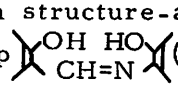
Meanwhile, the inhibitory effects *in vitro* of OK-432 or/and 5-fluorouracil on macromolecular syntheses in L1210 cells were tested on the one hand, and spleen cells in culture of mice pretreated with OK-432 were examined for uptake of ^{3}H-thymidine with or without stimulants, PHA and Con A, on the other. Unexpectedly, none of the data obtained were sufficient to account for the enhancement of antileukemic effect by OK-432 *in vivo*. The role of OK-432 as an immunopotentiator was further investigated, and the details will be presented in the other pages of this publication. Attempts are also being made to obtain the active principles, which are capable of inhibiting tumor growth *in vivo*, from live Group A streptococcal cells.

(45) Screening for new class of potent antitumor agents. 1) Structure-activity relationships for 2-(2-hydroxy-5-*n*-hexyl)-8-quinolinol-4-carboxylic acid and its analogous.

T. Ujiie and S. Koshimura

Intending to cultivate a new type of antitumor agent, several kinds of ligands and nitrofurans which were originally synthesized in this laboratory have been evaluated for their antitumor activities, resulting in discovery of some excellent compounds. For example, 2-(2-hydroxyphenyliminomethyl)-4-*n*-hexylphenol (HP) and 2-(2-(5-nitro-2-furyl)vinyl)quinoline (NQ) were shown to be effective against some kinds of experimental rodent tumors. Further efforts have been made as follows.

First, the modification of NQ by introducing N, N-diethylaminoalkyl-oxy or -amino group into quinoline ring of its chemical structure gave a series of compounds which were more water-soluble than the original compound and were able to form complexes with nucleic acids *in vitro*. Among them, 2-(2-(5-nitro-2-furyl)vinyl)-8-(β-(N, N-diethylamino)ethoxy)quinoline was shown to be active against ascites and solid forms of Ehrlich carcinoma, Sarcoma 180 solid tumor, mouse leukemia SN 36 and ascites hepatoma AH 13, but not to be effective on ascites hepatoma AH 66, Yoshida sarcoma and lymohoid leukemia L1210.

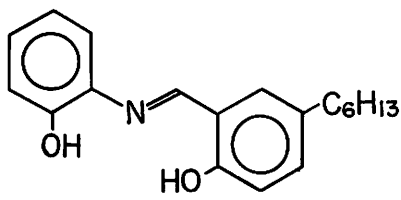
Second, in the studies on structure-activity relationships of HP, the existence of a group  (tridentate ligand) and an alkyl group (C_5 - C_8) in its molecule has revealed to be essential for the exhibition of biological activities. These findings followed study of a new active compound, 2-(2-hydroxy-5-*n*-hexyl)-8-quinolinol-4-carboxylic acid (HQ II), which has the functional groups described above and is stable in water.

Third, with regard to the mode of action of HP and HQ II, the

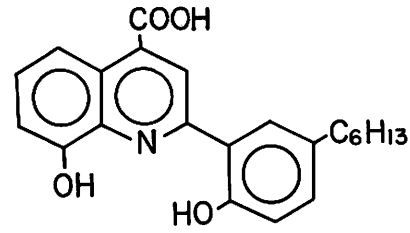
following results were obtained: Both compounds directly injured the membranes of animal tumor cells and of rabbit red cells depending on temperature *in vitro*. Furthermore, they selectively inhibited DNA synthesis in some kinds of eucaryotes including AH 13 cells and cultured HeLa cells in the presence of serum at the appropriate concentration. More detailed studies using AH 13 cells in culture indicated that the primary site of action of HQ II might be ribonucleotide diphosphate reductase (RDP reductase), which is an iron-requiring enzyme dealing with the reduction of RDP to dRDP and is a rate-limiting enzyme of DNA synthesis.

Meanwhile, three authorized antitumor agents, hydroxyurea, guanazole, and 5-hydroxypyridine-2-aldehyde thiosemicarbazone (5-HP), which possess the ability to form iron-chelate complexes and are the inhibitors of RDP reductase, have been known to selectively kill the cells during S-phase. In this respect, studies on cell killing kinetics by HQ II were carried out with the following results: Dose-surviving fraction curves obtained by plotting of numbers of formed colonies of HeLa cells which were previously exposed to the agent suggested that the mode of killing was phase specific and time dendent. Furthermore, cell synchronization at the G_1/S boundary phase occurred in cells which had been exposed to HQ II (70 μM). When the cells were treated at the higher concentration of HQ II, transient retardation of cell cycle progression from G_2-phase to M-phase and elongation of the G_1-phase was observed.

From these results, it seemed that HQ II might be a compound belonging to "S-phase specific with self-limiting" drugs which have been proposed by Skipper *et al.*



HP



HQ II

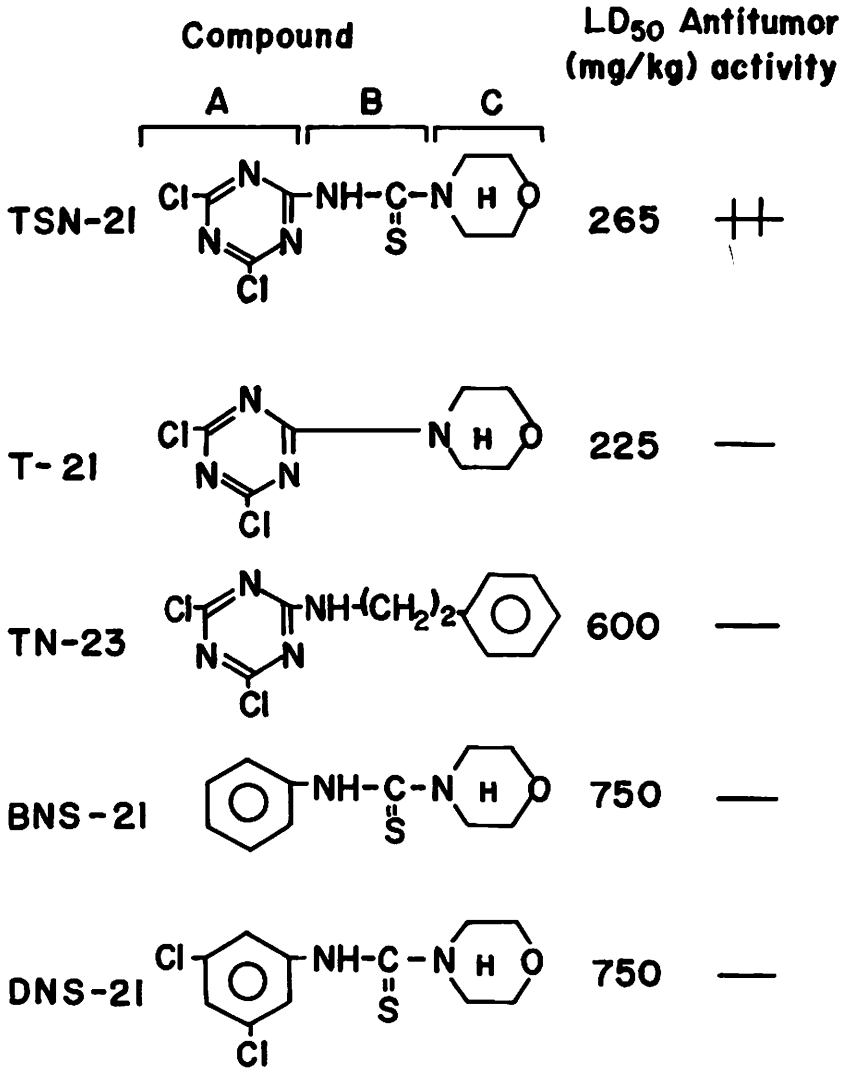
2) Antitumor effect of N-(2, 4-dichloro-*s*-triazinyl)-N′-diethylene oxide thiourea and its analogous.
S. Koshimura, T. Ujiie and H. Hirota

In vivo screening experiments to search the synthetic candidates for new antitumor agents resulted in one hundred or more of the *s*-triazine derivatives being tested for their antitumor activity, employment of various animal tumors, such as Ehrlich carcinoma, Sarcoma 180, lymphatic leukemia L1210 in mice, and Yoshida sarcoma, ascites hepatoma AH 13 in rats. Up to the present, experimental data have shown that N-(2, 4-dichloro-*s*-triazinyl)-N'-diethylene oxide thiourea (TSN-21) was found to

be most active; intraperitoneal administration of TSN-21 (80 mg/Kg, Days 1-7) to mice bearing Ehrlich ascites carcinoma or Sarcoma 180 ascites tumor produced a marked prolongation of life-span comparable to that of mitomycin C-treated mice. This compound was also effective against AH 13, but not effective or very slightly so against Yoshida sarcoma and L1210 leukemia.

Meanwhile, the results obtained from *in vitro* assay indicated that TSN-21 did not lyse rabbit red cells and Ehrlich tumor cells in concentrations of 10 mM and 2 mM, respectively, and the uptake of ^{3}H-thymidine and ^{3}H-uridine into Ehrlich tumor cells was suppressed to some extent (60-65%) at the concentration of 100 μg/ml. Investigations on structure-activity relationships re-

TSN-21 AND ITS ANALOGUES



vealed that the existence of dichloro-*s*-triazine and thiourea moieties in the chemical structure was found to be essential for the manifestation of antitumor activity, as shown in the figure. TSN-21 was shown to be of relatively low toxicity for animals; for example, by intraperitoneal injection of this compound, LD_{50} was 265 mg/Kg for male mice.

(46) The biological properties of coelomic fluid of the sea urchin.

K. Ryoyama and S. Koshimura

In the experimental series designed to seek antineoplastic substances from marine sources, the coelomic fluid of the sea urchin was previously found to possess the capacity to lyse and agglutinate red blood cells. This fluid had also exhibited damage *in vitro* against various transplantable tumor cells. The susceptibility of tumor cells to the coelomic fluid was clearly different

THE CORRELATION BETWEEN CELL SURFACE AREA AND CELL INJURING ACTIVITY OF COELOMIC FLUID PREPARATION FROM SEA URCHIN
(*A. Crassispina*)

Cell	Diameter (μ)	Surface Area* (μ^2)	EC 50**	$\times 10^{-6}$ μg /100 μ^2	Index***
YS	11.6±1.6	423± 8	57	5.4	1
YS Thio-TEPA	14.6±2.6	672±21	>7145	>425.3	<79
YS MMC	14.9±2.8	695±24	1312	75.5	14
YS 6-MP	13.1±2.0	537±13	443	33.0	6
YS 5-FU	14.1±2.4	627+18	≧648	≧41.3	≧8
AH 13	16.6±1.9	863±12	63	2.9	0.5
66F	17.3±1.8	934+10	406	17.4	3
109A	13.0±1.7	527+ 9	191	14.5	3
S-180	14.8±1.9	686±11	172	10.0	2
Fhrlich	15.9±2.5	794±20	102	5.1	0.9
L1210	10.2±1.4	325± 6	220	27.1	5
Rabbit Red cell	–	110****	3.3	1.2	0.2

* Surface Area = π x(Diameter)^2
** μg protein/5x106 cells
*** Calculated by the value(x10^{-6}μg/100 μ2) of YS cell as 1.
**** Cited from Condrea, E., et al. (1964), Biochim. Biophys. Acta 84, 365.

depending upon the species of tumor cells, suggesting that the differences may be due to the cell surface area of the tumor cells tested. By means of gel chromatography, the tumor cell-damaging activity of the fluid was found to have occurred in the fraction with hemolytic activity, but was not found in the hemagglutinating fraction and the others. Purification steps monitored by Sephadex column chromatography have indicated that the nature of its active factor may be a protein or a protein-like substance.

Meanwhile, the drug-resistant cell line of Yoshida sarcoma was found to be generally less susceptible to the coelomic fluid than the original sarcoma cell. Especially, the thio-TEPA-resistant cell line was more than 100 times resistant to the fluid. The results of analytical absorption tests with the cytoplasmic membrane fraction isolated from tumor cells revealed that the site of action of the fluid seems to be the locus of the cell membrane. Thus, it was thought that the differences in susceptibility to the coelomic fluid between the thio-TEPA-resistant cell line and the original Yoshida sarcoma cell were not due to the inactivation of active factor(s) in the fluid, or to differences in the course of cell death caused by the fluid, but may be due to the amounts of receptor site on both cells against active factor(s) of the coelomic fluid. The insusceptibility of this drug-resistant cell line to the coelomic fluid was found not to be dependent on the cell surface area.

In this respect, we are now studying: 1) active factor(s) in the coelomic fluid of the sea urchin, 2) active sites of tumor cell membrane which reacts with the factor, 3) change in chemical composition and biological behavior of the tumor cell membrane in the process of acquiring drug-resistance.

(47) Studies on the streptococcal hemolysin formed in the presence of colistin.

M. Fujita and S. Koshimura

Evidence has been previously presented in this laboratory to show that when colistin, a basic peptide antibiotic, was added to the reaction mixture of β -hemolytic streptococci and oligoribonucleotide in Bernheimer's basal medium (BBM), there occurred a marked increase of hemolytic activity ($2\text{-}5 \times 10^6$ hemolytic units/ml) in the medium, as compared with that ($1\text{-}2 \times 10^4$ hemolytic units/ml) in colistin-deficient medium. Such a phenomenal manifestation was obtained by addition of neither colistin alone nor oligoribonucleotide alone to the coccal suspension.

The ability of the cocci to form colistin-induced hemolysin (CIH) was sustained in the cells grown at late exponential phase in the culture medium containing 0.01% sodium thioglycollate. The strains of β -hemolytic streptococci capable of producing streptolysin S (SLS), Su and Blackmore, were found to be useful in acquiring CIH, while C203U strain, which produces only streptolysin O, was ineffective in this respect.

An oligoribonucleotide, rich in guanylic residue, separated by

DEAE-cellulose column chromatography could be favorably compared with RNase core in production of CIH in the medium. The yield of this hemolysin was fully dependent upon the concentration of oligoribonucleotide (50-100 μg/ml) and colistin (100 μg/ml) respectively, and also upon the concentration of K^+ (100 mM), $Mg2^+$ (10 mM) ions and maltose (5-10 mM) contained in the reaction medium. When one of the three components was omitted, CIH was not obtained, even though colistin and oligoribonucleotide were present.

Effect of various chemicals on hemolysis by F-I and F-II

Chemical	Concentration (μg/ml)	Hemolytic activity (HU/ml)		
		F-I	F-II	SLS*
Trypan blue	-	800	832	810
	6	98	<10	<10
	0.6	230	25	23
Lecithin	-	255	349	387
	40	30	34	47
	8	74	87	80
Cholesterol	-	800	832	810
	320	<10	963	868
	32	12	915	896
Cysteine**	-	1,766	1,727	1,702
	500	1,459	1,280	1,248

* Purified streptolysin S preparation.
** After preincubation with cysteine at 37C for 10 minutes, hemolytic activity was estimated.

This hemolytic activity detected in the medium was completely abolished by trypan blue and partially by cholesterol. Colistin itself exhibited no hemolytic effect on red blood cells and had no influence on hemolysis by SLS.

The fractionating diagram of crude CIH preparation by DEAE-cellulose column chromatography indicated the presence of two peaks with uniform distribution of hemolytic activity and optical density at 260 nm; i.e., one (F-I) eluted immediately after the first chromatographic run, and the other (F-II) later at a salt concentration of 0.6 M. F-II was similar to SLS in all respects of its response to various chemicals and in physical properties, as shown in Table. It was, furthermore, presented to show that the activity of F-I, which was not adsorbed by the anion exchanger, was abolished by trypan blue and lecithin as well as SLS, and also suppressed completely by cholesterol, unlike SLS.

The results obtained in these experiments revealed, at least, that one of the hemolysins produced by hemolytic streptococci in the presence of colistin and oligoribonucleotide might be a potent unknown hemolysin other than SLS and SLO. Very recently, a

lyophilized crude CIH preparation was separated by gel filtration on Sephadex G-100 into two segments with hemolytic activity.

The proceedingly eluted fraction on CM-Sephadex column chromatography was detected for a single segment which is defferent from the SLS moiety, indicating maximum UV absorption at 278 nm.